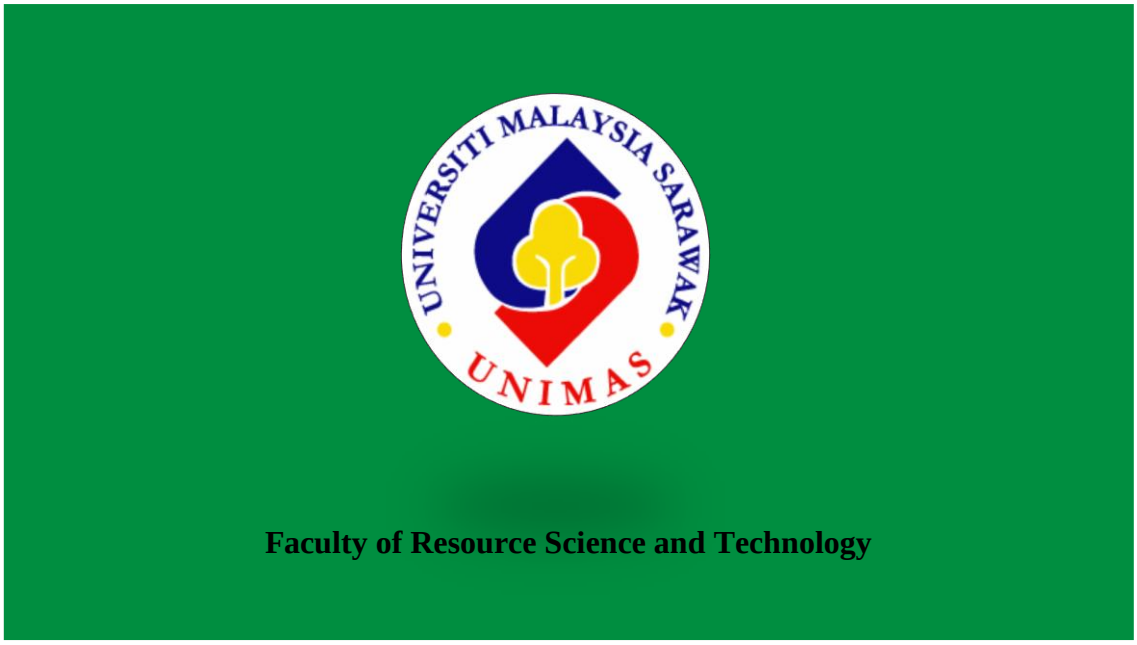

Prevalence of *Vibrio cholerae* and *Vibrio parahaemolyticus* in wild birds, rodents and bats from Nanga Merit, Kapit.

Evlyn Lemok Anak Noel

Bachelor of Science with Honours
Resource Biotechnology
2010

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LIST OF ABBREVIATION

–	Negative
+	Positive
%	Percent
^{o}C	Celcius
µl	Microlitre
g	Gram
ml	Mililitre
mm	Milimeter
spp.	Species
G	Green
P	Purple
W	White
Y	Yellow
APW	Alkaline Peptone Water
CT	Cholerae toxin
CV	CHROMagar^{TM} Vibrio
IT	Indole test
LBB	Luria Bertani Broth
KIA	Kligler Iron Agar
H_2S	Hydrogen sulphite
NaCl	Sodium chloride
MR	Methyl Red
MRVP	Methyl Red-Vogues Proskuer
MSA	Mannitol Salt Agar
NG	No Growth
OT	Oxidase test
SCT	Simmons citrate test
TCBS	Thiosulphite Citrate Bile Salt
TSA	Trypticase Soy Agar
TSI	Triple Sugar Iron
Tdh	Thermostable direct hemolysin
Trh	Thermostable related hemolysin
V. cholerae	*Vibrio cholerae*
V. parahaemolyticus	*Vibrio parahaemolyticus*
VP	Vogues-Proskuer

Prevalence of *Vibrio cholerae* and *Vibrio parahaemolyticus* in wild birds, rodents and bats from Nanga Merit, Kapit

EVLYN LEMOK ANAK NOEL

This report is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honors in Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2010

DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of as application for another degree of qualification of this or any other university or institution of higher learning.

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Prevalence of *Vibrio cholerae* and *Vibrio parahaemolyticus* in wild birds, rodents, and bats from Nanga Merit, Kapit.

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ABSTRACT

Vibrio cholera and *Vibrio parahaemolyticus* are two common *Vibrio* species to cause illness to human. The common symptoms caused by these pathogens are vomiting, blood stool, fever, headache, and watery diarrhoel. In this project, the objectives were to isolate these two bacterial species from Nanga Merit samples and compare prevalence of these organisms in birds, rodents and bats. The Alkaline Peptone Water (APW) containing 1% NaCl was used to enriched the samples. CHROMagar™ Vibrio. CHROMagar™ Vibrio was used to isolate *V. cholerae* and *V. parahaemolyticus*. Thiosulphite Citrate Bile Salt (TCBS) agar was used to compare the colony growth and appearance from CHROMagar™ Vibrio. From thirty samples, only ten samples showed desired colony growth for *V. parahaemolyticus* and none for *V. cholerae* on CHROMagar™ Vibrio. These presumptive *V. parahaemolyticus* isolates undergone eight biochemical tests. However, none of the samples showed positive result for all biochemical tests. This study indicates a low risk of bacteria a being transmitted from the animal to the environment.

Keywords: Alkaline Peptone Water (APW), Nanga Merit,*V. cholera, V. parahaemolyticus*, CHROMagar™, biochemical tests.

ABSTRAK

Vibrio cholera dan *Vibrio parahaemolyticus* merupakan dua species yang kerap menyebabkan keracunan makanan terhadap manusia. Simptom-simptom penyakit yang biasa disebabkan oleh dua pathogen ini adalah muntah, najis berdarah, demam, sakit kepala dan cirit berair. Objektif projek ini adalah untuk mengenalpasti kedua-dua jenis bakteria dari sampel Nanga Merit dan membandingkan taburan kehadiran organisma ini dalam burung, roden, dan kelawar. Air Peptone Beralkali mengandungi 1% NaCl digunakan untuk memperbanyakkan sampel. CHROMagar™ Vibrio. CHROMagar™ digunakan untuk proses pengasingan *V. cholerae* dan *V. parahaemolyticus*. Thiosulphite Citrate Bile Salt (TCBS) digunakan untuk membandingkan pertumbuhan dan keadaan koloni dari CHROMagar™ Vibrio. Daripada tiga puluh sampel, hanya sepuluh sampel yang menunjukkan pertumbuhan *V. parahaemolyticus* and tiada untuk *V. cholerae* di atas CHROMagar™ Vibrio. Sepuluh sampel ini kemudian menjalani lapan ujian biokimia dan semuanya negatif untuk semua ujian biokimia. Kajian ini menunjukkan bahawa risiko untuk bakteria dipindahkan daripada haiwan ke persekitaran adalah rendah.

Keywords: Alkaline Peptone Water (APW), Nanga Merit,*V. cholera, V. parahaemolyticus*, CHROMagar™, biochemical tests.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Genus *Vibrio* are Gram negative bacteria, straight or curved cell shaped, oxidase-positive for most species and having single polar flagellum (Todar, 2009). Pacini was a person who coined term *Vibrio* in 1854 and later changed the name of *Vibrio bacillus* to the *Vibrio cholerae* (Farmer III and Hickman-Brenner, 2006). This genus is placed under family Vibrionaceace which includes genus Photobacterium, Aeromonas, and Plesiomonas (Drasar, 1997). This genus is mostly found in water environment; brackish, saltwater, and freshwater environment and they widely distributed in marine environment (Farmer III and Hickman-Brenner, 2006). Most of the species are associated with marine organism. They are also pathogenic to other organism such as eels, frogs, vertebrate, and invertebrate (Todar, 2009). Therefore, *Vibrio* spp. is considered as water-borne disease.

Vibrio cholera and *Vibrio parahaemolyticus* are two common species often to cause illness to human. However, *V. cholerae* and *V. parahaemolyticus* have different way to infect human whereby *V. cholerae* infect small intestine through enterotoxin secrection while *V. parahaemolyticusis* an invasive organism as it infects the colon directly (Todar, 2009*). V. cholerae* produce cholera toxin to cause illness while *V. parahaemolyticus* produce hemolysin to cause illness in human. Haemolysin producing organism usually comes from Kanagawa phenomenon-positive strain (ESR, 2001).

Cholerae disease caused by *V. cholerae* had become endemic for long time ago dated back in 15th century. Robert Koch successfully isolated the *V. cholerae* from cholerae patient intestine in 1883 and suggesting the existence of cholerae enterotoxin (CT) in 1884 (WHO, 2002 and Todar, 2009). This disease became pandemic in 1961 to 1971. El Tor biotype is one of *V. cholerae* strain which became epidemic in 1961 in Phillipines. Between 1967 and 1974, the classical strain of *V. cholerae* replaced by El Tor strain for cholerae disease transmission in India. In 1991, outbreaks of El Tor biotype hit Peru after 100 years free from cholerae endemic. Subsequently, in 1991, a new strain emerged which is *V. cholerae* O139. This strain began to emerge in Bangladesh and this strain is derived from El Tor. *V. cholerae* O139 strain had changed its antigenic structure from El Tor type and people lived in endemic area were susceptible to this strain during that time.

Illness caused by *V. parahaemolyticus* is usually related to raw seafood consumption. It is seafood-borne pathogen and this pathogen had caused outbreaks in 1950 at Japan (Kiiyukia *et. al.,* 1989). This pathogen first identified in Japan and illness is associated with consuming shrimp and later outbreaks spread to other seafood such as crab and prawn (Drasar, 1997). The incident of food-borne cause by *V. parahaemolyticus* increased in 1997 and 1998 in Japan, which caused by serotype O3:K6 clone (Bhuiyan *et. al.,* 2001). The diseases that caused by *V. parahaemolyticus* and *V. cholerae* is gastroenteritis. The common symptoms caused by these two pathogens are vomiting, blood stool, cramps nausea, fever, headache and watery diarrhoeal. For *V. parahaemolyticus*, the incubation period for symptoms to show off is between 12 to 24 hours and *V. cholerae* is two hours to five days.

In Malaysia, there were about 1672 cholerae patients found in Sarawak from 1994 and 2003 and this endemic hit Sarawak during El Nino season in 1997 and 1998 (Benjamen, 2006). The outbreaks in Sarawak occurred in Northern region which are Miri, Bintulu and Limbang. Majority of cholerae patient was normal adult and low-income labours were the group that susceptible to this disease (Benjamen, 2006).

1.2 Objectives

The aims of this study were:

i. To isolate *V. cholerae* and *V. parahaemolyticus* from Nanga Merit samples on CHROMagar™ Vibrio (CV) and Thiosulphite citrate bile salt (TCBS) agar.

ii. To identify the *V. cholerae* and *V. parahaemolyticus* isolates through series of biochemical tests.

iii. To compare the prevalence of *V. cholerae* and *V. parahaemolyticus* in wild birds, rodents and bats from Nanga Merit.

CHAPTER 2

LITERATURE REVIEW

2.1 *Vibrio* species

Genus *Vibrio* is belonging to family Vibrionaceae. The general characteristics for organisms belong to genus *Vibrio* are short rods either curved or straight rod and the size of the cell is 0.5 x 3 µm (Drasar, 1997). The bacterial cells linked from one end to one end. The organisms under this genus cannot form spore. This genus can grow under aerobic and anaerobic condition; and grow under alkaline condition which is between pH 8 to 9.5 (Drasar, 1997). *Vibrio* spp. require simple medium for growth. The addition of 1-3% NaCl stimulates the growth of *Vibrio* in medium (Drasar, 1997 and Todar, 2009). This genus generally grows at ambient environment. The optimum temperature for their growth is between 18-37 ^{o}C and they can tolerate environment pH ranging from pH 6.0 to 9.0 (Drasar, 1997).

2.2 *Vibrio cholerae* and *Vibrio parahaemolyticus*

Vibrio cholerae and *Vibrio parahaemolyticus* are common species among other vibrio species to cause illness. Both species can grow in simple medium like peptone water. *V. cholerae* can grow in medium with or without addition of salt (Lake *et. al.,* 2003; Choopun *et. al.,* 2002; Rhodes *et. al.,* 1985). According to Rhodes *et. al.,* (1985), there were growth for *V. cholerae* in 0%, 3% and 5% NaCl and inhibited at 7% NaCl. Choopun *et. al.,* (2002) reported that the bacterial growth was variable, which means that some *V.*

cholerae strains were able to tolerate salt content more than 5% and some not. For *V. parahaemolyticus*, it is a halophilic organism. They are able to survive in water containing high salt content. They can survive NaCl content slightly higher than *V. cholerae* but cannot grow in medium not supplemented with NaCl. The optimum temperature for both bacteria to grow is 37 ^{o}C and both organisms can grow in aerobic condition or anaerobic condition but they grow best in aerobic condition. The optimum pH for the bacterial species is pH 7.8 to 8.6.

2.3 Strains and disease

V. cholerae have more than 150 strains but the strains that lead to illness are O1 serotype and O139 serotype (Drasar, 1997). The common strain which cause illness by *V. parahaemolyticus* is known as positive Kanagawa phenomenon (Drasar, 1997). *V. parahaemolyticus* differ to *V.cholerae* whereby the strains which more likely to cause illness is the combination of serotype of O antigen and K antigen. Serotype O3:K6 is the *V. parahaemolyticus* serotype that was responsibled for gastroentritis in India (Bhuiyan *et. al.*, 2001).Toxin associated to cholerae is cholerae toxin (CT). For *V. parahaemolyticus*, it produces enterotoxin and *tox*R gene is responsible in disease (Bhuiyan *et. al.*, 2001). Hemolysin is the virulence factor in *V.parahaemolyticus* illness. There are two genes involved in *V. parahaemolyticus* disease which are thermostable direct hemolysin (*tdh*) and thermostable related hemolysin (*trh*) genes. For cholerae endemic, it mostly occurs in country or place that has poor sanitary system or inadequate clean water supply (Kumar

et.al., 2009). For *V. parahaemolyticus,* the major cause for illness is consuming uncooked or raw seafood.

2.4 Media

2.4.1 CHROMagar™ Vibrio

CHROMagar™ Vibrio was developed by Hara-Kudo and team in University of Tokyo. This agar has improved the detection especially for *V. parahaemolyticus.* Detection of *V. cholerae* and *V. vulnificus* has also improved using this agar. The colony colour for *V. cholerae* and *V. parahaemolyticus* on CHROMagar™ Vibrio is violet and blue-green, respectively. The principle behind the use of CHROMagar™ is based on the used of chromogenic substrate where the media contained substrate for beta-galactosidase enzymatic activity (Hara-Kudo *et. al,* 2001). The use of this agar is found to be time saving and save labour cost especially to detect *V. parahaemolyticus* as its colony colour does not share with other bacteria colony colour (Hara-Kudo *et. al,* 2001). According to Hara-Kudo *et. al,* (2001), the violet color *V. parahaemolyticus* did not change when left at room temperature up to 18 hours. This violet colour colony also is not affected by other bacterial colony grown on CHROMagar™ Vibrio. The violet and blue-green colour colony generated on agar depends on bacterial beta-galactosidase reaction with substrate in CHROMagar™ Vibrio (Hara-Kudo *et. al*, 2001). They reported that, *V. parahaemolyticus* found to be more frequently isolated on CHROMagar™ Vibrio compared to TCBS(Nissui Co., Tokyo Japan) and TCBS(Oxoid Unipath Ltd, United Kingdom) (Hara-Kudo *et. al,* 2001).

2.4.2 Thiosulphite Citrate Bile Salt Agar (TCBS)

TCBS agar is formulated by Kobayashi *et. al*.(1963). This agar is beige in color in powder form. It becomes green in color when it is in liquid and solid form. TCBS agar contained bromthymol blue which used as indicator for pH changes. Combination of sodium thiosulphite with ferric ammonium citrate in this agar is to detect hydrogen sulfide organism. Oxgall added in this agar formula intended to inhibit Gram-positive bacteria. TCBS agar is initially to isolate *Vibrio* spp. from clinical and non clinical samples. *V. alginolyticus* and *V. cholerae* appear to be yellow colonies when grown on this agar. There are some limitations of using this agar. Firstly is that *V. paramahaemolyticus* colonies are confused with *Aeromonas hydrophilla*, *Pseudomonas* spp., and *Plesiomonas shigelloides* for first isolation on the agar. Second, some late fermenting sucrose of *V. cholerae* strains appear green and colourless colonies and may resemble to *V. parahaemolyticus*. Third is, *Proteus* spp that ferment sucrose may be confused with *Vibro* spp. in which they grow as yellow colonies.

2.4.3 Mannitol Salt Agar Phenol Red (MSA)

Mannitol salt agar contains 7.5% sodium chloride, mannitol, nutrient agar and phenol red. This agar can be used to test salt tolerance organism. Phenol red intended to use as indicator whereas any organism that ferment mannitol will grow as yellow colonies (Hammond, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Samples Collection

Samples from disturbed area was collected by a team of researcher from Microbiology Lab from Universiti Malaysia Sarawak during a field trip to a remote village in Nanga Merit, (15-25th June 2009) Kapit. The cloacal, anal and intestine swabs were collected from the birds, rodents and bats using sterile cotton buds. They were immediately placed into 900µl of phosphate buffer saline and stored at 4 ^{o}C throughout the field trip. The samples were brought back to the UNIMAS Microbiology laboratory to be processed.

Eleven and nineteen samples of cloacal swabs were choosen from the samples collected from human settlement areas (Site 1) and forest area (Site 2) respectively. Details of the samples are as shown in Table 1, Table 2, and Table 3.

Table 1: Samples from Nanga Merit Site 1 and 2

Nanga Merit Site Origin	Organism	Number of samples
Site 1	Birds	4
	Rodents	1
	Bats	5
	Unknown	1
Site 2	Birds	8
	Rodents	0
	Bats	10
	Unknown	1
Total		30

Table 2: Sources of samples from site 1 (human settlement site)

Sample code	Local name	Scientific name
A2791(F)	Red headed tailor bird	Not available
U2119(I)	Small woolly bat	*Kerivoula intermedia*
U2116(I)	Small woolly bat	*Kerivoula intermedia*
U2118(I_2)	Short-nosed fruit bat	*Cynopterus brochytis*
U2122(I)	Not available	Not available
DO2602(I)	Emerald dove	*Chalcophaps indica*
DO2602(F)	Emerald dove	*Chalcophaps indica*
U2118(A)	Short-nosed fruit bat	*Cynopterus brochytis*
U2117(A)	Muller's rat	*Sundamys muelleri*
U2119(A)	Small woolly bat	*Kerivoula intermedia*
A2789(C)	Purple naped sunbird	*Hypogramma hypogrammicum*

Table 3: Sources of samples from site 2 (pristine area)

Sample code	Local name	Scientific name
FNM091(A)	Lesser wolly horseshoe bat	*Rhinolophus sedulus*
CO3377(A)	Spotted-winged Fruit Bat	*Balionycteris maculata*
AO9011(C)	Chestnut-winged Babbler	*Stachyris erythoptera*
CO3363(A)	Fawn roundleaf bat	*Hipposideros cervinus*
CO3358(A)	Fawn roundleaf bat	*Hipposideros cervinus*
FNM060(I)	Spotted-winged Fruit Bat	*Balionycteris maculata*
AO9067(C)	Little Spiderhunter	*Arachnotera longirosta*
FNM050(F)	Bornean horseshoe bat	*R. borneensis*
AO9043(C)	Short-tailed Babbler	*Malacocincla malaccensis*
BO9712(C)	Hairy-backed Bulbul	*Tricholestes criniger*
AO9064(C)	Olive-winged Bulbul	*Pycnonotus plumosus*
CO5301(F)	Not available	Not available
FNM063(A)	Fawn roundleaf bat	*Hipposideros cervinus*
AO9052(C)	Little Spiderhunter	*Arachnotera longirosta*
FNM050(A)	Emerald dove	*Chalcophaps indica*
AO9060(C)	Little Spiderhunter	*Arachnotera longirosta*
CO3365(C)	Fawn roundleaf bat	*Hipposideros cervinus*
FMN080(A)	Fawn roundleaf bat	*Hipposideros cervinus*
FNM151(I)	Short-nosed fruit bat	*Cynopterus brochytis*

3.2 Isolation of *Vibrio* species

3.2.1 Enrichment of bacteria samples

The samples were enriched in Alkaline Peptone Water (APW) containing 1% NaCl. 50 µl from each sample was enriched in 2 ml APW and incubated for 18-24 hours at 37 ^{o}C. This enrichment step was performed according to Farmer III and Brenner (2006); Choopun *et. al.*, (2001) and Lesmana *et. al.*, (1997).

3.2.2 Selective isolation of *Vibrio* on CHROMagar^{TM} Vibrio

After overnight incubation, the enriched bacteria samples streaked onto CHROMagar^{TM} Vibrio (CV) plates (CHROMagar^{TM} Microbiology, France). CV was prepared according to manufacturer instruction. Later, these plates were incubated 18-24 hours at 37 ^{o}C. The morphology of the colonies on the agar were observed and recorded. Purple or violet colonies formed were picked and streaked on Tryticase Soy Agar (TSA) slant containing 3% NaCl for working cultures. Table 4 below was used as reference to identify *V. parahaemolyticus* colonies on CV.

Table 4: Colony morphologies of various bacteria grown on CV medium and TCBS medium

Species	No of strains tested	CV medium		TCBS medium	
		Size of colony (mm)	Colour of colony	Size of colony	Colour of colony
Citorobacter freundii	1	NG		NG	
Edwardsiella tarda	1	NG		NG	
Enterobacter cloacae	2	NG		NG	
Escherichia coli O157:H7	2	NG		NG	
Klebsiella ornithinolytica	1	NG		NG	
Klebsiella oxytoca	1	NG		NG	
Photobacterium damselae	1	NG		NG	
Proteus mirabilis	2	Minute	Milk White	Minute	Blue Green
Providencia rettgeri	1	1	Milk White	Minute	Blue Green
Pseudomonas aeruginosa	1	NG		NG	
Salmonella enteritidis	2	NG		NG	
Serratia marcescens	1	NG		NG	
Shigella sonnei	2	NG		NG	
Vibrio alginolyticus	4	5-6	Milk White	3-4	Yellow
Vibrio cholerae O1	2	3	Pale Blue	3	Yellow
Vibrio hollisae	1	4	Milk White	3	Green
Vibrio mimicus	2	3-4	Pale Blue	1-2	Green
Vibrio parahaemolyticus	68	3-5	Violet	2-4	Green
Vibrio vulnificus	1	5	Pale Blue	1	Green

(Hara-Kudo *et. al.,* 2001)